

HIGH SALINITY ACCLIMATION BY THE PRAWN *MACROBRACHIUM ROSENBERGII*: UPTAKE OF EXOGENOUS AMMONIA AND CHANGES IN ENDOGENOUS NITROGEN COMPOUNDS

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ABSTRACT

The freshwater prawn *Macrobrachium rosenbergii* was subjected to a hyperosmotic transfer from 0‰ to 24‰ salinity. Changes in ammonia excretion, blood Na^+ , pH, protein, free amino acids (FAA), and ammonia were monitored for 48 h. Following a rapid reduction in ammonia excretion after transfer, ammonia concentrations in exposure water declined for 24 h. These losses could not be attributed to bacterial growth or aerial diffusion. Also during the first 24 h, blood Na^+ increased from 150 to 280 mM, still far below ambient Na^+ concentrations of 366 mM. Blood ammonia decreased nearly five-fold and protein concentrations in blood declined about 40 g/l. These data may indicate a reversal of normal $\text{Na}^+/\text{NH}_4^+$ exchange following a hyperosmotic shock, such that blood Na^+ is hyporegulated using exogenous NH_4^+ as a counter-ion. During the first 24 h after transfer, net ammonia acquired by uptake could be used to increase intracellular ammonia concentrations as a prelude to increased synthesis of FAA. This model may complement recent evidence of a $\text{Cl}^-/\text{HCO}_3^-$ reversal in fish hyporegulating blood Cl^- in seawater.

INTRODUCTION

The mechanisms of osmoregulation in euryhaline invertebrates have been studied and reviewed extensively (Schoffeniels, 1976; Kirschner, 1979; Gilles, 1979). Extracellular osmolarity depends primarily on excretory and extrarenal mechanisms of ion and water regulation (Prosser, 1973), whereas intracellular osmolarity depends on changes in the concentration of intracellular free amino acids (FAA) and other low molecular weight nitrogenous solutes (Gilles, 1975; Bowlus and Somero, 1979).

Like many euryhaline organisms, crustaceans exposed to low salinity environments maintain blood osmolarity and ionic concentrations hyperosmotic and hypertonic to the external medium (Kerley and Pritchard, 1967; Siebers *et al.*, 1972; Mangum *et al.*, 1976). Upon transfer to high salinity environments, blood ionic and osmotic concentrations increase due to efflux of water and inward movement of ions. Cell volume during hypersaline stress is regulated by an increase in intracellular FAA. Concomitant with this is a decrease in ammonia excretion, thought to indicate greater use of endogenous ammonia to synthesize amino acids (Mangum and Towle, 1977; Gilles, 1979).

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Abbreviations: FAA, free amino acids; FW, freshwater; EC, extracellular, IC, intracellular.

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The research discussed here concerns the osmoregulatory response of the freshwater prawn *Macrobrachium rosenbergii* subjected to a hyperosmotic shock. *M. rosenbergii* was chosen for this study because of its euryhaline distribution in nature (Johnson, 1967; George, 1969); its tolerance of large salinity changes, as evidenced by strong osmoregulation (Sandifer *et al.*, 1975; Armstrong, unpublished data); and its wide commercial use, for which physiological information may be helpful in culture programs. Of interest were changes in blood and tissue nitrogenous compounds and blood inorganic ions; in particular, changes in patterns of ammonia excretion after freshwater-acclimated animals were transferred to saltwater. The results reported here indicate that during the early phase of acclimation to a hyperosmotic shock, *M. rosenbergii* apparently takes ammonia from the surrounding medium.

The functions of ammonia in osmoregulatory processes have been studied mainly in two respects: first as a constituent of FAA for intracellular osmotic regulation (Siebers *et al.*, 1972; Bishop, 1976); and second as a counter-ion for regulation of blood Na^+ (Evans, 1975; Maetz *et al.*, 1976; Mangum and Towle, 1977; Kirschner, 1979). Previous studies of these two processes have considered the role of ammonia only in terms of endogenous pools. Apart from its toxicity in solution (Campbell, 1973; Colt and Tchobanoglous, 1976; Armstrong *et al.*, 1978) ammonia is linked to osmoregulation only as an internal molecule that an animal somehow must void. Based on results of this study, we propose two complimentary hypotheses that examine the possible uptake of exogenous ammonia for osmoregulatory functions during and following high salinity acclimation.

MATERIALS AND METHODS

Animals

M. rosenbergii juveniles were reared from larvae of second and third generation University of California, Davis, brood stock. About 60 additional animals were obtained from the hatchery of R. Yates, Winters, California. Broods were mixed and mass reared in 400-l freshwater (FW) tanks equipped with biological filtration. Animals were held in this FW system at least 2.5 months before experiments. Their diet consisted of Purina Ration #20, frozen adult *Artemia*, and fish. Animals' weights and lengths differed somewhat within groups and between the groups of different experiments. Average dry weight of more than 220 experimental animals was 0.436 ± 0.081 (standard deviation) g, with a range from 0.131 to 1.120 g. Carapace length (orbit of eye to posterior of carapace) averaged 12.6 ± 0.7 mm and ranged from 8.5 to 18.0 mm. Temperature and pH in rearing tanks averaged 25°C and 8.10. Terminology for ammonia speciation used throughout this report is that of Colt and Tchobanoglous (1976) and Armstrong *et al.* (1978): "ammonia" refers to the sum of $\text{NH}_3 + \text{NH}_4^+$; NH_3 signifies un-ionized ammonia; and NH_4^+ the protonated, ionized form.

Experimental protocol

Approximately 1 week before excretion experiments, we removed animals from large rearing tanks, placed 15 in each experimental freshwater 40-l aquarium at 27°C, and subsequently fed the animals twice daily. Ambient ammonia was consistently low during this time (0.0 to 3.0 μM) and total nitrite was $<0.43 \mu\text{M}$ (well below concentrations found stressful to larvae of this species; Armstrong *et al.* 1976). From 24 h before experiments began and for their duration, animals were

not fed. Time without food therefore ranged from 48 to 72 h depending on the length of tests.

Two salinities were tested for their effect on excretion: 0‰ (FW), and 24‰ made with tap water and Instant Ocean salts. Water stood in bulk overnight before tests; the pH of both salinities averaged $8.28 \pm .07$ (not significantly different) and was very stable throughout experiments. On the morning of a test, NH_4Cl was added to both salinities to a total ammonia concentration of $30 \mu\text{M}$ ($=510 \mu\text{g/l}$). These solutions were then measured into test beakers. This concentration is higher than levels in many FW lakes, streams, or near-shore and pelagic oceanic areas (Hutchinson, 1957; Lee and Boda, 1977), but it is not atypical of values in eutrophic estuaries and their lowland rivers. For instance, ammonia concentrations in the Sacramento-San Joaquin Delta of California range from 2.6 to $26.5 \mu\text{M}$ $\text{NH}_4\text{-N}$, with values up to $70 \mu\text{M}$ at some stations (State of California, 1976). *Macrobrachium* is endogenous to fresh and brackish water habitats of the Indo-Pacific (Johnson, 1967; George, 1969), feeding in the flooded, organically rich rice plains of India and Bangladesh. An ammonia concentration of $30 \mu\text{M}$ is probably representative of that in the prawns' natural habitat.

To measure ammonia excretion, shrimp were thoroughly rinsed in de-ionized water and placed one per 250 or 400 ml beaker (containing 200 or 300 ml of water, respectively). Generally, 0.420 g dry wt was the maximum size of animals put in 250 ml containers. Half the beakers contained FW, half 24 ‰ seawater. There was no prior acclimation of animals to the high salinity. This salinity shock is not lethal (Armstrong, unpublished observations). Each beaker was covered with a plastic petri dish penetrated by a glass pipette from an air manifold. Aeration was gentle and constant throughout all tests. Partial immersion in a bath maintained water temperature at 27°C .

A 2.5 ml water sample, taken from each beaker within 1 min after animals were introduced, was considered time zero (T_0). Subsequently, water from each beaker was generally sampled at 1, 2, 4, 8, 12, 16, and 24 h, and at various times up to 48 h during longer tests. For 48 h experiments water was changed completely at 15 and 33 h, with appropriate T_0 samples taken after each change. In this way, ammonia excretion of 40 animals per experiment was monitored through as many as seven time intervals in 24 h. Ammonia in water samples was immediately reacted with phenol-hypochlorite (Solorzano, 1969) and colorimetrically quantified with a Perkin-Elmer 550 spectrophotometer. Newly molted or soft shrimp were not used in experiments, and the data from animals that molted during tests were not included in the analyses presented here.

In a series of experiments designed to measure the ion regulatory ability of *M. rosenbergii*, groups of animals at 27°C were acclimated to salinities between 0‰ and 28‰. Salinity was increased 4‰ per day and each group was kept at its final salinity 48 h before blood was sampled. Time course changes in blood Na^+ and NH_4^+ after a hyperosmotic shock were studied by abruptly transferring groups of animals from 0‰ to 24‰. Blood samples were collected from groups of 8 to 15 shrimp in as many as eight time periods over 48 h; blood was never taken more than once from any animal.

Analytical procedures

Blood samples: About $10 \mu\text{l}$ of hemolymph was drawn directly from the pericardial sinus of animals sampled as different experimental groups. This sample was transferred to 2.5 ml glass-distilled water and ammonia determined as previously

outlined. Sodium was measured in blood transferred to 3 ml of double glass-distilled water containing 1% HNO_3 , 0.2% La, and 500 ppm Cs; protein was precipitated and removed by centrifugation. Ion concentrations were determined with a Varian Techtron 1200 atomic absorption spectrophotometer. Blood pH was determined *in vivo* by inserting a combination microelectrode (Microelectrode, Inc., MI-410) directly into the pericardial sinus, taking care not to puncture the hepatopancreas or stomach. Protein concentration and free amino acids were measured from the pellet and supernatant, respectively, after 24 h extraction and 20 min centrifugation (10,000 g) of blood transferred to 85% ethanol at 0°C. Protein was redissolved in 0.5N NaOH and assayed by the method of Lowry *et al.* (1951). Primary amines were quantified on a Turner Model 111 fluorometer after reaction with fluorescamine (North, 1975), using a glycine standard. Total osmolality was measured on a Wescor 5100 B osmometer, calibrated with NaCl, by transferring blood directly to the machine without clotting or freezing; it was assumed that proteins contributed an insignificant portion of the osmotic pressure.

Tissue: Time course changes in tissue FAA were measured by sampling tail muscle from groups of 20 shrimp killed at various times during 48-h excretion experiments. Muscle portions were blotted, dried 48 h at 50°C, weighed, and stored no longer than 2 weeks in stoppered Nalgene vials until analyzed. Dried tissue was pulverized and extracted in 85% ethanol with intermittent shaking for 24 h at 0°C. Samples were then centrifuged at 10,000 g for 20 min in the cold, and primary amines were assayed from the supernatant as previously described. Additionally, secondary amines were measured by reaction with isatin in the dark (Boktor, 1971) and were read immediately at 595 nm against a proline standard.

Calculations: Proportions of NH_3 were calculated as described by Armstrong *et al.* (1978); the $\text{pK}'a$ used for 24‰ was 9.33 (see Colt and Tchobanaglou, 1976; Armstrong *et al.*, 1978 for reviews of $\text{pK}'a$ calculations) and for FW was 9.15.

Data for ammonia, FAA, and proteins were obtained on a dry weight basis or per volume of blood. To convert data to other units the following relationships were used: Total body water of shrimp in FW and 24‰ seawater was $71.9\% \pm 0.6$ (2.56 g water·g dry wt⁻¹) or $72.1\% \pm 0.8$ (2.61 g water·dry wt⁻¹) respectively (no significant difference; Armstrong, unpublished data). A representative value of 72% (2.57 g water·dry wt⁻¹) was used. Of the total body water, 22% was assumed to be extracellular (EC), based on several studies that measured this parameter in crustaceans (Kerley and Pritchard, 1967; Binns and Peterson, 1969; Lang and Gainer, 1969; Spaargaren, 1972), leaving 78% as intracellular (IC) water. Further, we assumed these values were constant over time after the high salinity transfer, although this is an oversimplification required by lack of specific data.

Results of excretion experiments were compared by analysis of covariance to determine if there was a negative weight-specific relationship, as reported in other studies (Bartberger and Pierce, 1976; Nelson *et al.*, 1977). Analysis of variance was used to test the significant of other relationships, such as change in blood pH or ammonia concentration over time as a function of salinity transfer.

Controls

Ammonia could be lost from the water during excretion experiments by three routes: (1) uptake by the shrimp, (2) uptake by bacterial populations increasing over time, and (3) aerial diffusion to the atmosphere. Tests to gauge loss by the latter two routes were as follows: Aerial loss of ammonia was measured by adding autoclaved FW or 24‰ water (pH 8.25, no significant change during tests) plus

30 or 140 μM ammonia to 10 beakers each (total of 40), constantly aerated as previously described. Water samples were taken at T_0 and every 12 h thereafter to 48 h. The rate of loss as $\mu\text{moles ammonia} \cdot \text{h}^{-1}$ was calculated by a least squares regression of ammonia concentration vs. time.

Growth of bacteria during tests and their possible contribution to changes in ambient ammonia concentrations were gauged by direct plate counts from the medium through time, and use of antibiotics. Excretion experiments were performed as described, by placing shrimp individually in FW or 24‰ beakers (200 ml volumes) to half of which 30 mg chloramphenicol/l and 20 mg streptomycin/l had been previously added (no prior acclimation of animals). Ammonia was added to beakers for initial concentrations of 6.1 and 140 μM . A series of water samples were taken to measure ammonia excretion and subsamples were pooled for bacterial plating. A standard dilution series of pooled water was plated onto autoclaved peptone beefheart extract in agar, made either with FW or 24‰ (Instant Ocean salts). Colonies were counted after 36 h incubation at 27°C. The number of bacteria/ml calculated, times the volume of water in beakers gave the total number of bacterial cells in the water column through time. These values was multiplied by the factor 2×10^9 cells/mg dry wt (Stanier *et al.*, 1970) to obtain dry bacterial biomass in micrograms. Finally, an average dry weight nitrogen content of 15% (Stanier *et al.* 1970) was used as a multiplier to get micrograms and micromoles of bacterial nitrogen. Assuming that all bacterial growth during experiments was based on autotrophic use of ammonia nitrogen (ignoring heterotrophic use of organics), then changes in bacterial nitrogen over time may reflect the quantity of ammonia lost via bacterial uptake.

RESULTS

All control shrimp in FW beakers survived throughout all tests, including eight animals that molted during this time. Animals abruptly transferred to 24‰ also survived, and showed no gross signs of stress. In nearly all cases there was no loss of equilibrium, and animals at both salinities appeared equally active. Several shrimp in 48-h tests developed slight opacity in tail muscle (see Sindermann, 1977, for discussion of stress related to this condition), but none died, even though 10 animals molted during tests (an event likely to amplify any stress due to salinity shock).

Rates of ammonia excretion differed significantly in FW and 24‰ groups ($P < 0.01$, two-way ANOVA) despite sometimes high variability of rates from one time interval to the next, even within a single experimental group. Since analyses of covariance indicated an equivocal relationship between weight-specific ammonia excretion and total dry weight (significant in some time intervals but not in others), rates here are without covariate corrections.

Average rates of excretion of animals in FW were always positive from one time interval to the next, although their magnitude varied considerably during the first 8 h of tests (Fig. 1). Between 0–1 h, about $5.0 \mu\text{moles ammonia} \cdot \text{g dry wt}^{-1} \cdot \text{h}^{-1}$ were excreted, a rate that had declined to $0.2 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ by the second hour. The consistently very high excretory rate in the first 1 or 2 h probably resulted from stress from netting and washing when animals were placed in beakers. After 8 h in a beaker, excretion stabilized between 0.95 and $1.40 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Fig. 1).

Transfer to high salinity markedly changed ammonia production. Excretion in the first hour was about $0.75 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, or about 15% of the rate of ammonia

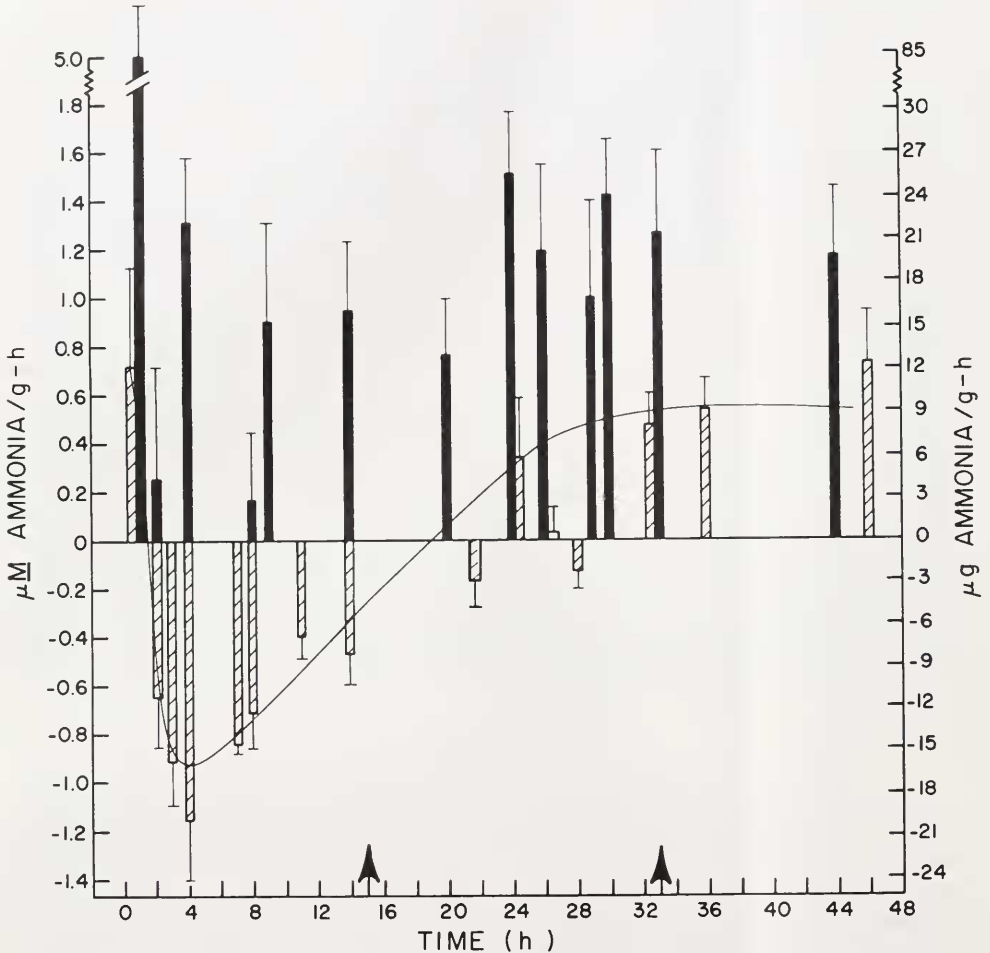


FIGURE 1. Rate of ammonia excretion or uptake by *M. rosenbergii* in FW (solid bars) or transferred to 24 ‰ (hatched bars). Bar height is the mean $\pm$ SE of 20–80 animals per time period, based on dry weight. Each rate was calculated in the time interval between it and the preceding bar; for instance, the FW rate at 24 h is the interval between 20–24 h. The solid line represents the trend in ammonia excretion and uptake in 24‰. Arrows indicate water changes.

production by the FW group for this period. About 1 h after the transfer, excretory rates became negative (Fig. 1), as concentrations of ammonia in the medium progressively declined. This apparent uptake reached $1.6 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ in the 2–4 h interval, approximating the average excretion rate of FW animals between 4 and 48 h. Uptake continued for about 20 to 24 h, gradually declining from 1.16 to 0.7 and $0.5 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, at 4, 8, and 14 h, respectively (Fig. 1). Ammonia concentrations in beakers began to increase 24 h after the hyper-saline transfer, indicating animals had resumed net excretion of ammonia, although at a rate of $0.52 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, less than half that of FW controls (Fig. 1). Absolute quantities of ammonia lost from beakers during uptake phases, calculated and normalized to body weight, in the 24 h following a hypersaline transfer averaged $12.5 \mu\text{moles ammonia} \cdot \text{g dry wt}^{-1} \cdot \text{h}^{-1}$.

The ammonia uptake could have represented net losses to the atmosphere and bacteria during periods of very low, yet positive excretion by the shrimp in 24‰ salinity. Controls, however, showed this was not the case. Water of pH 8.25 lost ammonia at a rate of $-0.012 \pm 0.024 \mu\text{moles} \cdot \text{h}^{-1}$ over 48 h (no shrimp, aerial diffusion control). Multiplying rates at 2, 4, and 8 h (Fig. 1) by 0.436 g dry wt (average weight), the resultant values are -0.283 , -0.506 , and $-0.305 \mu\text{moles} \cdot \text{h}^{-1}$ lost from beakers, some 23 to 42 times greater than the rate of loss to air without animals.

Nor could changes in bacterial biomass as a reflection of autotrophic assimilation of ammonia account for ammonia's disappearance over time (Table I). In water with ambient ammonia levels of 6.1 and 140 μM , increases in bacteria over 24 h represented a possible loss of 0.019 and 0.34 μmoles ammonia, respectively, far less than the net decline of about 12.5 $\mu\text{moles} \cdot \text{g}^{-1}$ shrimp calculated above. Also, the rates of greatest ammonia uptake occurred in the first 8 h after transfer, when the slopes of exponential bacterial increase were probably more gradual than in the latter part of the 24-h period, *i.e.*, proportionally less ambient ammonia would be used for bacterial growth early in the incubation than later.

Chloramphenicol and streptomycin completely eliminated bacteria from the water column by 4–9 h but had no significant effect ($P > 0.05$, ANOVA) on ammonia uptake between treated and untreated groups in 24‰ (Table II). The magnitude of uptake differed in two experiments at 6.1 and 140 μM ammonia, but there were no significant differences related to antibiotic treatment within an experiment (Table II). However, when experiments were done in FW with initial ammonia concentration of 6.1 μM , antibiotics significantly lowered ammonia excretory rates ($P < 0.01$, ANOVA) in all time intervals studied. For instance, between 4–8 h excretory rates were $0.85 \mu\text{moles} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ($\pm .23 = \text{SE}$) with antibiotics and 1.55 ($\pm .45$) without; and between 8–18 h were 0.63 ($\pm .29$) and 2.55 ($\pm .58$)

TABLE I

Increase in bacterial biomass in the water column through time during shrimp excretion experiments, and calculated ambient ammonia required to account for bacterial increases. Values represent eight pooled water samples at 24‰ salinity. Cell count:dry weight ratio was taken as 2×10^9 cells/mg dry wt, nitrogen content as 15% of dry weight. See text for details.

Elapsed Time (h)	Initial ammonia (μM)	No. bacteria/ml	No. bacteria/beaker	Bacteria dry wt. (μg)	Calculated ammonia use	
					μg	$\mu\text{moles} \times 10^{-3}$
0	6.1	—	—	—	—	—
	(110 $\mu\text{g/l}$)					
4	"	1,400	2.03×10^5	0.10	0.015	0.9
9	"	6,500	9.23×10^5	0.46	0.069	4.1
24	"	32,000	4.38×10^6	2.19	0.328	19.0
0	140	—	—	—	—	—
	(2500 $\mu\text{g/l}$)					
5	"	51,000	7.24×10^6	3.62	0.543	32.0
24	"	569,000	7.68×10^7	38.4	5.760	340.0

TABLE II

*Effects of antibiotics (30 mg chloramphenicol/l plus 20 mg streptomycin/l) on rates of apparent ammonia uptake by *M. rosenbergii* after transfer from FW to 24‰, and at different initial ambient ammonia concentrations. Rates are given for a common intermediate period (4–8 h), and as averages for entire tests. Negative signs indicate loss of ammonia from beakers (uptake), a positive value shows an increase (excretion).*

Initial ammonia (μM)	+/- Antibiotics	Number animals	Average uptake rates ($\pm$ SE) (μ moles ammonia $\cdot$ g dry wt ⁻¹ $\cdot$ h ⁻¹)		
			4–8 h	0–18 h	0–36 h
6.1	–	8	–.37 (.11)	.12 (.11)	
6.1	+	8	–.25 (.09)	–.14 (.05)	
140	–	16	–1.36 (.21)		–.41 (.19)
140	+	16	–1.13 (.12)		–.66 (.12)

respectively. If bacteria were using ammonia for growth, then apparent ammonia excretion should have increased or uptake decreased when they were eliminated. Yet either the reverse was true or there was no effect on excretion/uptake rates when bacteria were suppressed.

Concentrations of blood ammonia in the high-salinity group declined significantly ($P < 0.01$, ANOVA, $F = 102.7$, d.f. = 7, 113) by 4 h after transfer to 24‰. It continued to drop for 20 h, from an initial high of 4.82 mM to about 1.0 mM, eventually reaching 0.71 mM by 48 h (Fig. 2). During the same 48 h, blood ammonia of the FW group declined significantly, from 4.82 to 2.94 mM (Fig. 2); still, however, four times higher than in the high salinity group. Blood pH in both treatments increased significantly, although more so in the high salinity, where it rose from 7.58 to 7.87. Blood NH_3 ranged from 23 to 84 μM , while water NH_3 never exceeded 3 μM , indicating a large diffusional gradient from blood to water. NH_3 was 1.8–3.5% of the total blood ammonia and 7.3% of that in water, the balance being ammonium ion (NH_4^+), which was 41–148 times more concentrated in the blood than in water during 24 h incubation (Fig. 2).

Changes in blood concentrations of other nitrogenous compounds were also studied. Protein levels dropped precipitously within 3 h after a transfer to 24‰ salinity, from 107 g protein $\cdot$ l⁻¹ blood to 63 g $\cdot$ l⁻¹ (Fig. 3), a decline of 41% ($P < 0.01$, ANOVA, $F = 22.5$, d.f. = 4,35). Following this initial decline, concentrations in blood of animals at 24‰ remained constant at about 65 g $\cdot$ l⁻¹ through 27 h, while protein levels in blood of FW control shrimp increased slightly but insignificantly to 119 g $\cdot$ l⁻¹. Blood FAA changed only slightly over 10 h after transfer to high salinity ($P > 0.05$, ANOVA, $F = 2.04$, d.f. = 3,40) and did not parallel the declines in blood ammonia and protein. Total primary FAA increased from 3.45 to 4.25 mM $\cdot$ l⁻¹ blood and declined back to 3.5 mM $\cdot$ l⁻¹ by 10 h after transfer to 24‰ (Fig. 4).

Concentrations of both primary and secondary amines in tail muscle increased significantly 24 h after transfer to 24‰ ($P < 0.01$, ANOVA, $F = 14.00$, d.f. = 4,88) but appeared not to have reached steady states by 48 h (Fig. 5). Total primary amines increased from 570 to 678 mM $\cdot$ kg dry wt⁻¹ (glycine standard), while secondary amines doubled from 48 to 95 mM $\cdot$ kg⁻¹. The net increase of all amines was 155 mM FAA $\cdot$ kg dry wt⁻¹. Based on IC water, primary amines increased from 285 to 340 mM $\cdot$ l⁻¹ intracellular water, while proline rose from 23.4 to 47.5

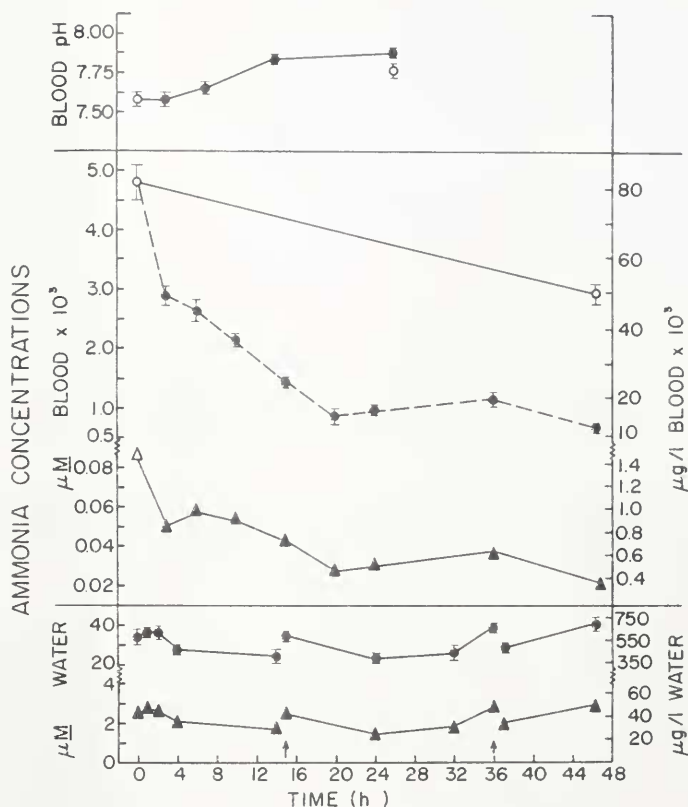


FIGURE 2. Change in concentrations of ammonia and pH values in blood of shrimp transferred from 0‰ to 24‰ (mean $\pm$ SE of 9 to 15 animals per interval). Total ammonia added to beakers at T_0 = 30 μ M. Mean water values are given for comparison to blood concentrations. For both water and blood, circles = total ammonia (NH_4^+ + NH_3), $\circ$ = 0‰, $\bullet$ = 24 ‰; triangles = un-ionized ammonia (NH_3). NH_3 was calculated as described in text, $\text{pK}'a$ = 9.33 for shrimp blood and ambient water at 24‰. Water pH constant at 8.23, blood pH beyond 26 h assumed to be 7.85.

$\text{mM} \cdot \text{l}^{-1}$; a total net gain in these two FAA groups of about $78 \text{ mM} \cdot \text{l}^{-1}$ intracellular water.

M. rosenbergii slowly acclimated to various salinities strongly regulated blood Na^+ concentrations in ambient waters from 0‰ to 24‰ (Fig. 6A). Animals hyperregulated Na^+ to an isotonic point of about 210 mM Na (≈ 14 ‰) and hypo-regulated Na^+ between salinities of 14–28‰. Over an ambient Na^+ concentration of 5 (0‰) to 366 (24‰) mM, average blood sodium increased from 150 to 250 mM.

After a transfer from 0‰ to 24‰, blood Na^+ and total osmolarity, initially very hypotonic to the new medium, began to increase in 2–3 h. The rate of net sodium increase in blood over the first 7 h was $12.1 \text{ mM} \cdot \text{h}^{-1}$, and between 7 and 48 h only $1.2 \text{ mM} \cdot \text{h}^{-1}$ (Fig. 6B). Forty-eight h after the transfer there was still a large hypotonic gradient between blood and water Na^+ (285 and 366 mM, respectively; Fig. 6B). The blood equilibration value of 285 mM Na^+ after abrupt transfer was very close to 260 mM when shrimp were gradually acclimated to 24‰ (Fig. 6A).

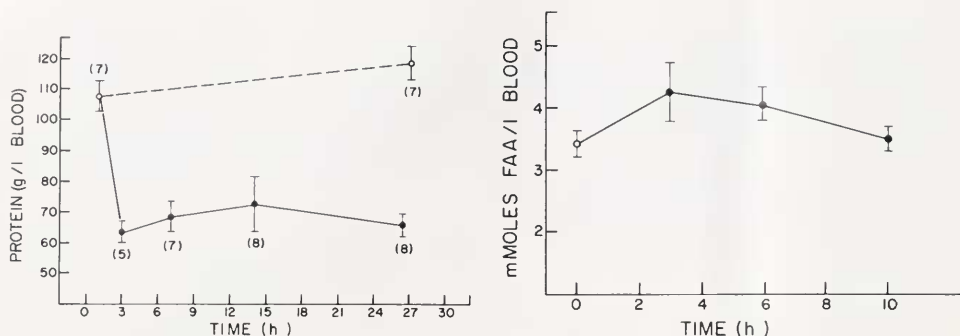


FIGURE 3. Concentrations of protein in the hemolymph of *M. rosenbergii* following a transfer from 0‰ to 24‰. Points are means $\pm$ SE, sample size in parenthesis. ○ = FW, ● = 24‰.

FIGURE 4. Concentrations of total primary amino acids in hemolymph of *M. rosenbergii* transferred from FW (○) to 24‰ (●). Points are means $\pm$ SE of 10–14 animals per time.

In contrast, total blood osmolarity increased $12.5 \text{ mOsm} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ and was isosmotic to 24‰ ($693 \text{ mOsm} \cdot \text{l}^{-1}$) 20 h after transfer (Fig. 6B).

DISCUSSION

Although an essential ingredient of many organic compounds, ammonia is usually viewed as a potentially toxic molecule, excreted from organisms as anabolic requirements are exceeded by catabolic release from assimilated food and by turnover of endogenous nitrogen compounds. Having once left an organism, ammonia *per se* is not thought to re-enter the bodies of higher aquatic animals except in toxic situations. We have shown in this study that when the euryhaline shrimp *M. rosenbergii* is subjected to a hyperosmotic shock, external ammonia decreases during the first 20–24 h after transfer to saltwater. This loss of ambient ammonia cannot be accounted for by bacterial uptake or aerial diffusion to the atmosphere,

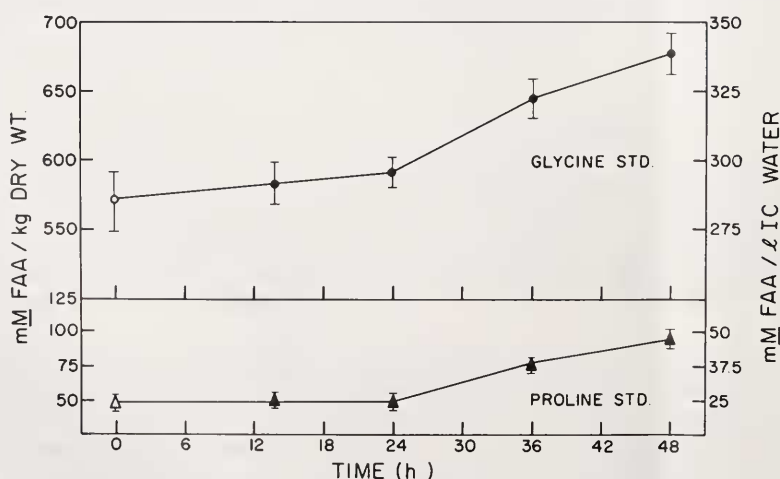


FIGURE 5. Concentrations of free amino acids in the tail muscle of *M. rosenbergii* acclimated to FW (open symbols) and following abrupt transfer to 24‰ (closed symbols) for 48 h.

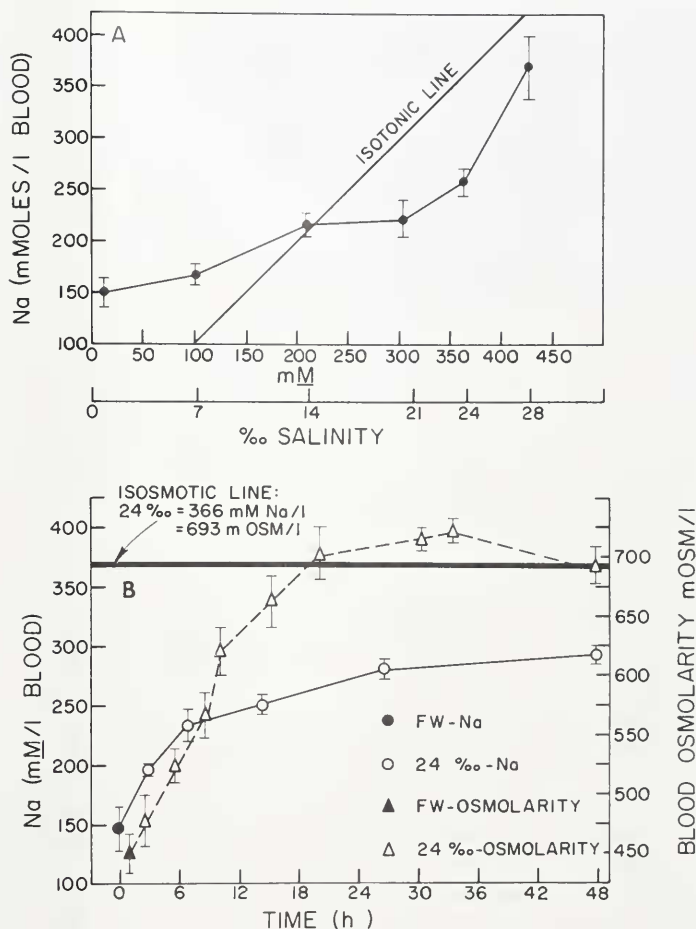


FIGURE 6. (A) Blood Na^+ concentrations in shrimp gradually acclimated to a specific salinity from 0‰ to 28‰. Animals were held at a given test salinity 48 h before sampling. Points are mean $\pm$ SE of at least eight animals per salinity. (B) Time course change in blood Na^+ and total osmolarity after an abrupt transfer from 0‰ to 24‰. Points are mean $\pm$ SE of data from 8–15 animals per interval.

and therefore indicates uptake by the animals themselves. This is the first report of ammonia uptake by higher aquatic animals, although several authors have suggested that possibility.

Maetz (1973) found that sodium influx to goldfish was inhibited by increased ammonia in the water, yet ammonia excretion was not. He concluded that an $\text{NH}_4^+/\text{NH}_3$ exchange was operative, but did not pursue its possible significance. During experiments to study the excretion of ammonia as NH_4^+ or NH_3 from marine invertebrates, Mangum *et al.* (1978) found that total ammonia of ambient water in vessels holding the clam *Rangia cuneata* declined from 5 to 2.44 mM during tests. Discounting loss by volatilization, they concluded that at least some ammonia permeated the body. Shaw (1960) reported the competitive inhibition of Na^+ influx into crayfish by NH_4^+ and postulated that this implied movement of the latter into animals. Thomas *et al.* (1976) reported enhanced growth of the gastropod *Biomphalaria glabrata* in alkaline water containing high concentrations of ambient

ammonia up to 75 mg/l. They hypothesized that NH_3 might diffuse into snails and serve a function in CaCO_3 deposition and/or act as a Na^+ counter-ion once protonated.

Changes in ammonia excretion following a hyperosmotic transfer have been studied in numerous crustacea (Jeuniaux and Florkin, 1961; Krishnamoorthy and Srihari, 1973; Mangum *et al.*, 1976). Although rates typically decline, excretion remains positive. Why, then, have previous studies not found evidence of transitory ammonia uptake as we report? Apart from intrinsic differences in the osmoregulator biochemistry between species, variations in experimental protocol may be the main reasons. Acclimation of animals to high salinities for several days to weeks often precedes measurement of ammonia excretion (Sharma, 1966; Krishnamoorthy and Srihari, 1973; Mangum *et al.*, 1976), thus giving animals sufficient time to equilibrate to a situation that may temporarily require ambient ammonia. In addition, samples may not have been taken frequently enough to observe temporary trends in ammonia fluxes, or excretion may have been based merely on two ammonia determinations over some period of time, so that the rate calculated is an average of trends during the interval (Needham, 1957; Sharma, 1966; Krishnamoorthy and Shrihari, 1973; Mangum *et al.*, 1976).

Possible osmoregulatory function of ammonia uptake: Na^+ regulation

Exogenous ammonia could have two possible osmoregulatory functions during acclimation to high salinity. The first deals with the poorly understood and apparently complicated branchial $\text{Na}^+/\text{NH}_4^+$ exchange in fish and crustacea (Kirschner *et al.*, 1973; Maetz *et al.*, 1976; Kirschner, 1979; Evans, 1980). Evidence indicates that freshwater aquatic organisms keep their blood Na^+ concentration hypertonic to ambient water by active uptake of external Na^+ , in part via exchange for blood NH_4^+ and/or H^+ . A number of recent studies indicate that seawater teleosts and elasmobranchs also possess $\text{Na}^+/\text{NH}_4^+$ exchange mechanisms (Payan and Maetz, 1973; Evans, 1975, 1977, 1980). Since seawater fishes maintain blood Na^+ concentrations hypotonic to the external environment, these exchange mechanisms impose an increased Na^+ load on the animal in addition to that incurred by drinking for water balance. Evans (1980) notes that the size of this load varies with species studied to date and may become appreciable in those organisms that maintain a branchial transepithelial potential close to that of the equilibrium potential for Na^+ .

Na^+ and NH_4^+ probably are exchanged across the branchial epithelium of freshwater-acclimated *M. rosenbergii*. A rapid transfer from FW (5 mM Na^+) to 24‰ (366 mM) immediately establishes a large hypotonic gradient between blood Na^+ (150 mM in FW) and the new medium. We tentatively suggest that during subsequent acclimation to 24‰, the direction of the $\text{Na}^+/\text{NH}_4^+$ pumps changes such that internal Na^+ is extruded in exchange for exogenous NH_4^+ (Na^+ blood/ NH_4^+ seawater). Although blood Na^+ increases to about 285 mM, it remains strongly hypotonic to the medium. It seems logical that an active pump, functioning to extrude Na^+ , would mitigate the increased diffusional influx. This putative reversal of $\text{Na}^+/\text{NH}_4^+$ exchange may be an important mechanism allowing the animal to hyporegulate blood Na^+ some 85 mM below ambient seawater.

Although we are aware of no previous work on the reversibility of the $\text{Na}^+/\text{NH}_4^+$ pump, De Renzis and Bornancin (1977) have demonstrated the existence of $\text{Cl}^-/\text{HCO}_3^-$ ATPase in fish, and Kormanik and Evans (1979) have provided evidence

for reversal of such a $\text{Cl}^-/\text{HCO}_3^-$ pump in high salinities. The $\text{Cl}^-/\text{HCO}_3^-$ exchange system of fish gill epithelia are as poorly understood as $\text{Na}^+/\text{NH}_4^+$ exchange, but most evidence indicates that in freshwater fish and crustacea, part of the Cl^- influx is coupled to efflux of HCO_3^- (De Renzis and Maetz, 1973; Ehrenfeld, 1974; Maetz *et al.*, 1976; Kirschner, 1979). In the seawater acclimated Gulf toadfish, however, Kormanik and Evans (1979) found that external HCO_3^- stimulated unidirectional Cl^- efflux with a K_m for HCO_3^- similar to normal seawater concentrations. This suggests that internal Cl^- , which is strongly hyporegulated, is exchanged for external HCO_3^- .

The proposed reversal of $\text{Na}^+/\text{NH}_4^+$ exchange during hyporegulation by *M. rosenbergii* in high salinity relies on the dual structure of ammonia. Active excretion of Na^+ can be linked to an ammonia cycle composed of exchange transport of exogenous NH_4^+ and back diffusion of NH_3 down a significant concentration gradient. In the current experiments, uptake of exogenous ammonia may greatly exceed ammonia excretion (diffusion) during the first 22–24 h, resulting in a net decrease in ammonia in ambient water. This is the period of greatest change in blood Na^+ concentrations, and may reflect a more extensive effort to control rapid Na^+ influx than is required after equilibration (Fig. 6B). Net ammonia gained in this period could be used in a second osmoregulatory function involving synthesis of intracellular FAA (see following section). During further acclimation to seawater (*i.e.* later than 24 h after transfer), net excretion may exceed uptake, possibly because of a less intense need to regulate Na^+ and/or because of other regulatory exchange mechanisms (such as Na^+ blood/ K^+ seawater) responding to the initial hyper-saline transfer.

The putative reversal of $\text{Na}^+/\text{NH}_4^+$ exchange also might continue hyporegulating blood Na^+ as a permanent feature of salt water acclimation. The NH_4^+ uptake- NH_3 diffusion cycle of this tentative model depends on intracellular branchial pH and total ammonia concentrations. Together, both factors determine the magnitude of the NH_3 gradient from cell to water. The pH difference *per se* probably will not favor diffusion outward, since ambient pH of our experiments was 8.28 and that of the blood about 7.62. IC pH of branchial cells would be a more likely factor, since this is the fluid first exchanged between the animal and the medium. For crustacea, IC pH in muscle may be 0.5–0.8 units lower than blood (Caldwell, 1954, 1958; Carter, 1972). Therefore, IC branchial pH might be 1.0 units less than the water of our tests, resulting in a 10-fold decrease in NH_3 on the acid IC side. Mangum and Towle (1977) depict a similar situation in *Callinectes sapidus* in high salinity, and suggest NH_3 diffuses from water to blood. However, the second factor to consider in anticipating the direction of NH_3 diffusion is total ammonia. In this regard, blood ammonia is not an appropriate measure as used in some calculations (Mangum and Towle, 1977; Armstrong *et al.*, 1978). Again, the branchial IC concentrations are the important pools of ammonia in discussions of $\text{Na}^+/\text{NH}_4^+$ exchange, and IC ammonia may be quite high.

Osmoregulatory studies have provided some fortuitous measures of ammonia in whole-body homogenates and isolated tissues of crustaceans, ranging from 2.2 to as high as 53 mM ammonia in IC water (recalculated from data of Gilles and Schoffeniels 1969a, b; Vincent-Marique and Gilles, 1970; Siebers *et al.*, 1972; assuming an average total body water content of 74% of wet weight and IC water equal to 78% of total body water). Blood ammonia, however, ranges from 0.05 to 1.0 mM (Gerard and Gilles, 1972; Siebers *et al.*, 1972; Mangum *et al.*, 1976). Thus, there is a 50-fold difference between extremes of the IC and blood ranges.

In molluscs, hemolymph ammonia is about 0.8 mM (Bartberger and Pierce, 1976; Strange and Crowe, 1979), while IC ammonia is about 10 to 20 mM and may be higher (recalculated from data of Shumway and Abbott, 1977, and Livingstone *et al.*, 1979; water content = 76% of wet wt and IC water assumed to be 78% of this value). From our tests, data on quantities of ammonia lost following the hyper-saline transfer have been calculated as concentrations per liter IC water (*see* "Materials and Methods"). Based on these calculations, IC ammonia could have increased by 7.8 mM 24 h after transfer. Add to this the quantities already present and that gained from blood, and IC ammonia for the entire animal could easily exceed 10 mM, as discussed previously for other invertebrates. (Since blood ammonia declined significantly after the hyperosmotic transfer (Fig. 2), extracellular fluids were not considered a probable sink for ambient ammonia lost during the first 24 h.) Again, considering that natural concentrations of ambient ammonia range from tenths μM to usually no more than 2-10 μM , the gradient of NH_3 from branchial cells to water may be several orders of magnitude, despite potentially unfavorable pH gradients.

Thus, the long-term (greater than 24 h) osmoregulatory response after a hyperosmotic transfer, and subsequent active hyporegulation of blood Na^+ , could include an exchange for ambient NH_4^+ , a portion of which continuously dissociates and rapidly diffuses back out the gills down a large concentration gradient. This in turn would result in a net excretion of ammonia, thereby preventing toxic buildup within the animal's body. Uptake of exogenous NH_4^+ in exchange for actively excreted Na^+ would compliment the model of $\text{Cl}^-/\text{HCO}_3^-$ reversal proposed by Kormanik and Evans (1979). Once transported from water to cell, NH_4^+ would dissociate to NH_3 , diffuse out (diffusion of NH_3 is the principle mechanism of ammonia excretion in blue crabs; Kormanik and Cameron, 1980), and leave H^+ to combine with the HCO_3^- exchanged for hyporegulated blood Cl^- . The uncharged H_2CO_3 could diffuse as is, or be converted to CO_2 and H_2O via carbonic anhydrase. Our model of reversed $\text{Na}^+/\text{NH}_4^+$ exchange is obviously tentative and awaits further experimental verification. A first study would measure ammonia and sodium fluxes during and after acclimation to high salinity.

Ammonia uptake: free amino acid synthesis

As discussed previously, ambient ammonia lost in the first 24 h after transfer could increase IC concentrations by as much as 7.8 mM. This rise in IC ammonia could stimulate GDH and simultaneously inhibit oxidative reactions of the Krebs cycle to maximize FAA production, as noted by Gilles and Schoffeniels (1969b). However, our data on tail muscle FAA concentrations show only a small increase within 24 h (Fig. 5). This suggests that either ambient ammonia does not contribute substantially to FAA synthesis, or that ambient ammonia is not distributed equally to the IC fluids of all tissues: Certain tissues such as nerves and gill epithelia may have to regulate cell volume rapidly, and hence may preferentially accumulate ambient ammonia for synthesis of amino acids.

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